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Staphylococcus aureus isolated from handmade sweets: Biofilm formation, enterotoxigenicity and antimicrobial resistance

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ABSTRACT

Staphylococcus aureus is the second most important pathogen involved in foodborne outbreaks in Brazil. Because of their widespread distribution and biofilm forming ability, handmade sweets are easily contaminated with *S. aureus*. The aim of this study was to isolate and identify coagulase-positive staphylococci (CPS) from handmade sweets produced in Pelotas City/Brazil. The virulence potential was checked by evaluating the presence of the staphylococcal enterotoxin genes, *ica*A and *ica*D genes, the biofilm forming potential and antimicrobial resistance of the isolates. It was find just *S. aureus* among the CPS isolates. All the *S. aureus* isolates had biofilm forming ability on stainless steel and more than half of them on polystyrene surfaces. The majority of the isolates carried the *ica*A (66.6%) and *ica*D (58.4%) genes and some of them had the genes encoding enterotoxins A (33.4%) and B (16.6%). Furthermore, the majority of the isolates (83%) were resistant to at least one of the tested antimicrobials and multidrug resistance was observed in 8.4% of the isolates. The isolates had virulence potential, and half of them were enterotoxigenic. In addition, the ability of all the isolates to produce biofilms highlights the danger posed by these potentially virulent microorganisms persisting in food manufacturing environments.

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1. Introduction

The city of Pelotas (Brazil) is a center for the production and sale of handmade sweets due to its history of Portuguese colonization. Currently, the city is considered to be the Brazilian sweet capital, and commercially produces a large quantity throughout the year, around 150.000 units/month (Canever et al., 2004). However, the sweets are mainly produced manually and, as a consequence, they may be easily contaminated with *S aureus*, either by the handlers or by biofilms formed on the equipment and utensil surfaces used in their preparation. *Staphylococcus aureus* is a Gram-positive coccus that is a non-motile and non-spore-forming facultative anaerobe. They are mesophilic and produce enterotoxins at temperatures between 10 °C and 46 °C. It is the second most important pathogen involved in outbreaks of foodborne disease in Brazil (Brazil, 2013; Trabulsi and Alterthum, 2008).

Staphylococcus aureus virulence is a multifactorial process that involves the production of a variety of cell-bound and secreted proteins, and biofilm formation. The classical staphylococcal enterotoxins (A, B, C, D and E) are responsible for 95% of cases of food poisoning outbreaks, and enterotoxins A and C are the most common (Al-Tarazi et al., 2009).

The biofilm forming ability of *S. aureus* is related to the presence of the *ica* operon, which is responsible for the production of the intercellular adhesion polysaccharide (PIA). Among the genes of this operon, *ica*A and *ica*D, are reported to be the most important for this function (Vasudevan et al., 2003). However, evidence is now emerging of the existence of *ica*-independent biofilm mechanisms capable of mediating intercellular accumulation in both *S. aureus* and *S. epidermidis* (O'Gara, 2007). In addition, a number of proteins not related to *ica*-dependent mechanisms have been implicated in biofilm formation (Giaouris et al., 2015).

Biofilms reduce the effectiveness of sanitizers, cause economic

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losses to industry, contaminate food and can increase the level of antimicrobial resistance (Mah, 2012; Simões et al., 2010).

The aim of this study was to isolate and identify coagulase-positive staphylococci from handmade sweets produced in Pelotas City, Brazil. After isolation and species identification, the aim was to check the virulence potential of the isolates by testing for the presence of *ica*A, *ica*D, the staphylococcal enterotoxin genes, and to study their biofilm forming potential and antimicrobial resistance profiles.

2. Material and methods

2.1. Isolation and phenotypic confirmation of coagulase-positive staphylococci

Fifty samples of handmade sweets produced in Pelotas City/Brazil were analyzed for the presence of CPS. The samples were collected and transported to the Food Microbiology Laboratory at the Federal University of Pelotas, where the analysis was conducted. CPS isolation was performed using the methodology described by the American Public Health Association (APHA, 2002). 25 g of fresh samples were homogenized in 225 mL of 0.1% peptone water (Acumedia®). Serial dilutions were made, and 0.1 mL samples were inoculated on Baird–Parker agar (BPA, Himedia®) Petri dishes and incubated at 37 °C for 48 h. Typical CPS colonies were identified by the free coagulase production test (APHA, 2002).

The isolates that had the capacity to produce coagulase *in vitro* were submitted to the acriflavine (Sigma Aldrich®) resistance test, using BPA, supplemented with acriflavine (7 μg mL^{-1}). The isolates were placed in Brain Heart Infusion broth (BHI, Acumedia®) and incubated at 37 °C for 24 h. An inoculum was streaked onto plates containing BPA plus acriflavine and incubated at 37 °C for 48 h. The characteristic result was the appearance of two halos around the colonies. This test was performed for all the CPS species, once, only *S. aureus* is resistant to acriflavine (Devriese, 1981).

2.2. Molecular confirmation of the isolates

2.2.1. DNA extraction

Genomic DNA was extracted according to the protocol recommended by Matthews et al. (1997) with modifications. A CPS culture on Tryptone Soy agar (TSA, Acumedia®) was transferred to 100 μL of TE solution (10 mM hydroxymethyl aminomethane - TRIS and 5 mM ethylenediamine tetra acetic acid – EDTA) until a turbidity 1.0 on the McFarland scale (1–2 colonies). Cell wall lysis was achieved by the addition of 100 μL of lysostaphin (100 μg/mL, Sigma Aldrich®) with incubation at 37 °C for 45 min, followed by the addition of 20 μL of TE-B buffer (50 mM hydroxymethyl aminoethane-TRIS and 20 mM ethylenediamine tetra acetic acid-EDTA) with SDS (20%) (sodium dodecyl sulfate, Invitrogen®), and 3 μL of proteinase K (2 mg/100 μL, Invitrogen®), and incubation at 37 °C for one hour. Then, 200 μL of NaCl (5 M) (sodium chloride, Synth®) was added and mixed gently for 15 s followed by centrifugation (10.000 × *g*, 4 °C, 15 min). The upper layer was transferred to a sterile tube and extracted with phenol: chloroform: isoamyl alcohol (25:24:1, Synth®) by centrifugation (16.000× *g*, 4 °C, 15 min). DNA was precipitated with two volumes of 95% cold ethanol and left to settle overnight (−20 °C). After centrifugation (16.000× *g*, 4 °C for 10 min) the pellet was washed twice with ethanol (70%) and dried for 30 min. It was suspended in 30 μL of ultra-pure water. Then, 2 μL of RNAse (10 mg/mL, Invitrogen®), was added and the mixture incubated at 37 °C for one hour. The DNA was maintained at −20 °C and quantified by spectrophotometry in an Eppendorf BioSpectrometer kinetic® (Eppendorf).

2.2.2. Multiplex PCR (mPCR)

Multiplex PCR was performed to identify the species of CPS, targeting the 16S rRNA gene for genus identification, and the *nuc* gene encoding the enzyme thermonuclease (one set of primers for each species: *S. aureus* - *nuc* 1, 2; *S. intermedius* – *nuc* 5, 6; and *S. hyicus* – *nuc* 7, 8). The primers used and programs are shown in Table 1.

The reaction mixtures contained 12.5 μL of GoTaq® Green Master Mix 2x (Promega Corp.), 1 μL of each primer at a concentration of 10 pmol for the thermonuclease gene and 1 pmol for the 16S rRNA gene, 2 μL of DNA (50 ng) and 2.5 μL of ultra-pure water (Promega Corp.) to a total volume of 25 μL. The mixtures were placed in an MJ Research® PTC 100.

The strains used as positive control for the reaction, negative controls for bacterial species identification and negative control for genus identification are shown in Table 2.

The PCR products were subjected to electrophoresis at 80 V for 70 min on a 1.5% agarose gel in 0.5x TBE buffer (Tris solution, boric acid-EDTA) using a molecular weight marker of 1 Kb (Invitrogen®). The amplified products were visualized by UV trans-illumination (Loccus®, L-Pix Touch).

2.3. Ability to form biofilms

The capacity for biofilm production was evaluated on polystyrene surfaces (PS) and stainless steel (SS) at 25 °C.

Biofilm forming ability on PS was assessed using 96-well microtiter plates, in accordance with the protocol proposed by Stepanović et al. (2007).

The optical density (OD) of each stained well was measured at 570 nm using an ELISA Plate Analyzer reader microplate (Robonick®, readwell PLATE). The negative control used was 0.85% saline water, and *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538 were used as positive controls.

Treated SS circular coupons (AISI 304 – 18% chromium and 8% nickel, combined with a maximum of 0.08% carbon, 8 cm in diameter) were used to evaluate biofilm forming ability. The coupons were washed with soft bristle brushes, neutral detergent and distilled water. After rinsing, they were immersed in acetone for 24 h to remove residual fat. Then, they were dipped in 0.1 N NaOH solution for one hour, rinsed with distilled water (3 times) and subjected to an ultrasound bath for one hour (Unique®, Model 1400 A, 40 kHz). Finally, prior to use, the coupons were individually packaged and sterilized.

The isolates were tested in duplicate as proposed by Rieu et al. (2008), with modifications. To evaluate biofilm formation on SS, the coupons were placed in Petri dishes containing Tryptone Soy broth (TSB, Acumedia®) supplemented with 1% glucose. The isolates were inoculated at a concentration of 0.5 on the McFarland scale (1/100,~10^8 CFU/mL), followed by cultivation at 25 °C for 24 h. Subsequently, the culture medium was removed and the coupons were washed twice with 10 mL of PBS buffer (Laborclin®) to remove loosely adherent cells. They were then dried for 24 h at 25 °C. Then strongly adherent (i.e. biofilm) cells were removed by streaking sterile swabs dipped in 0.85% saline solution over their surfaces, and vortexing them for 1 min. Ten-fold serial dilutions were made in duplicate, and plated on TSA, followed by incubation for 24 h at 37 °C.

2.4. Statistical analysis

Biofilm formation data on PS and SS were submitted to statistical analysis of variance (ANOVA) using Tukey's test ($p < 0.05$) with STATISTICA 7.0 software (Statsoft, Tulsa, USA).

Table 1
Primers used for identification of coagulase-positive staphylococci and virulence genes.

Primer	Sequence 5′- 3′	pb	Program	Reference
NUC1	ATGAAGTCAAATAAATCGCT	458	P1	Baron et al. (2004)
NUC 2	TTTGGTGAAAAATACTTCTC			
NUC 5	GAAAAAAATTACAACAGGCG	106	P1	Gandra et al. (2011)
NUC 6	CACATCCGTTGAAGACTTTT			
NUC7	TAAGACACCGATAAAAGCCC	740	P1	Bastos (2008)
NUC8	TTGTTTTTGTGCTTGTTCTATAC			
16S1	GGACGGGTGAGTAACACGTGG	252	P1	Baron et al. (2004)
16S2	TCCCGTAGGAGTCTGGACCGT			
SEA1	ACGATCAATTTTTACAGC	544	P2	Rosec and Gigaud (2002)
SEA2	TGCATGTTTTCAGAGTTAATC			
SEB1	ATTCTATTAAGGACACTAAGTTAGGGGA	404	P2	Jarraud et al. (2002)
SEB2	ATCCCGTTTCATAAGGCGAGT			
SEC1	GACATAAAAGCTAGGAATTT	257	P3	Rosec and Gigaud (2002)
SEC2	AAATCGGATTAACATTATCCA			
SED1	CAAATATATTGATATAATGA	330	P2	Zocche et al. (2009)
SED2	AGTAAAAAAGAGTAATGCAA			
SEE1	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	P2	Jarraud et al. (2002)
SEE2	CACCTTACCGCCAAAGCTG			
*ica*A F	CCTAACTAACGAAAGGTAG	1315	P4	Vasudevan et al. (2003)
*ica*A R	AAGATATAGCGATAAGTGC			
*ica*D F	AAACGTAAGAGAGGTGG	381	P4	
*ica*D R	GGCAATATGATCAAGATAC			

P1= 4 min at 95 °C; 2 min at 95 °C, 2 min at 52.7 °C, 2 min at 72 °C for 32 cycles; 7 min at 72 °C; **P2** = 5 min at 95 °C, 1 min at 95 °C,1 min at 44.5 °C, 1 min at 72 °C) for 37 cycles 10 min at 72 °C; **P3** = 5 min at 95 °C; 45 s at 95 °C, 45 s at 46.2 °C, 45 s at 72 °C) for 35 cycles; 10 min at 72 °C; **P4** = 45 s at 92 °C, 45 s at 49 °C, 1 min at 72 °C for 30 cycles.

Table 2
Characterization of the species group strains and isolates used in this study.

Species	Strain	Source	Application
S. aureus	ATCC 25923	Clinical isolate	PC: mPCR (*nuc* 1, 2), *ica*A and *ica*D genes, Antimicrobial resistance and Biofilm formation tests
S. aureus	ATCC 6538	Human lesion	Biofilm formation test
S. intermedius	ATCC 29663	Pigeon nares	PC mPCR (*nuc* 5 and 6)
S. hyicus	ATCC 11249	Pig with exudative epidermitis	PC mPCR (*nuc* 7 and 8)
S. xylosus	ATCC 29971	Human skin	NC mPCR (bacterial species)
S. sciuri	ATCC 29061	Southern flying squirrel skin	NC mPCR (bacterial species)
S. lugdunensis	ATCC 49576	Clinical isolate	NC mPCR (bacterial species)
S. epidermidis	ATCC 12228	Clinical isolate	NC mPCR (bacterial species) and NC *ica*A and *ica*D
S. haemolyticus	ATCC 29970	Human skin	NC mPCR (bacterial species)
S. capitis	ATCC 35661	Human Skin	NC mPCR (bacterial species)
S. saprophyticus	ATCC 15305	Urine	NC mPCR (bacterial species)
S. simulans	ATCC 27851	Human skin	NC mPCR (bacterial species)
Escherichia coli	ATCC 11229	Clinical isolate	NC mPCR (genus)
S. aureus	FRI S6	Chicken	PC *sea* and *seb* genes
S. aureus	ATCC 19095	Leg abscess	PC *sec* gene
S. aureus	FRI 361	Chicken	PC *sed* gene
S. aureus	FRI 326	Chicken	PC *see* gene
SAS 1 - 12	This study	Handmade sweets	Antimicrobial resistance and Biofilm formation tests

PC: Positive control; NC: Negative control; ATCC- American Type Culture Collection; FRI- Food Research Institute.

2.5. Detection of icaA, icaD and classical enterotoxin genes

The PCR to evaluate the presence of genes *ica*A and *ica*D was performed according to the protocol proposed by Vasudevan et al. (2003), with adaptations. The reaction solution contained 12.5 μL of GoTaq® Green Master Mix 2x (Promega Corp.), 1 μL of each primer (10 pmol), 2 μL of DNA (50 ng) and 8.5 μL of ultrapure water (Promega Corp.) to a final volume of 25 μL.

Primers that target the genes coding for the classical staphylococcal enterotoxins (*sea, seb*, *sec, sed* and *see*) were used. The reactions were prepared as described above for the presence of genes *ica*A and *ica*D.

The positive and negative controls used in the reactions for *ica*A, *ica*D and the classic enterotoxin genes are shown in Table 2. The primers used, as well as the programs and applications, are shown in Table 1.

2.6. Antimicrobial resistance tests

Working cultures of the isolates were maintained on Tryptic Soy agar (TSA, Acumedia®), following incubation at 37 °C for 24 h. Inocula were obtained from overnight broth cultures adjusted to approximately 1,4 × 10^8 CFU/mL, a turbidity equivalent to a 0.5 McFarland standard. The inocula were spread on the surface of Mueller-Hinton agar (MHA) plates. The disk diffusion test, recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015), was employed to determine the susceptibility to 12 antimicrobials: gentamicin (10 μg), chloramphenicol (30 μg), clindamycin (2 μg), sulfonamide (300 μg), ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), vancomycin (30 μg), tetracycline (30 μg), penicillin (10 U), cephalothin (30 μg) and cefoxitin (30 μg) purchased from the company Laborclin®.

The results were interpreted in accordance with the standards

for inhibition zone diameters for *Staphylococcus* spp. (CLSI, 2015). *Staphylococcus aureus* ATCC 25923 was used as the positive control for the tests.

3. Results

3.1. Isolation and identification of coagulase-positive staphylococci

Of the fifty samples of handmade sweets, six (12%) were contaminated with CPS. Twelve isolates were selected (sample one – two isolates, sample two – three isolates, sample three – four isolates; sample four, five and six – one isolate) and designated SAS (*Staphylococcus aureus* sweet) 1–12.

All the isolates were resistant to acriflavine, and therefore phenotypically characterized as *S. aureus* (Capurro et al., 2010; Devriese, 1981; Matos et al., 1991).

After the molecular tests, all the isolates were confirmed to be *S. aureus*, since there was amplification of a 252 bp fragment related to 16S rRNA, specific to the *Staphylococcus* genus, and amplification of a 458 bp fragment produced by the *nuc* set of primers 1 and 2, specific to this microorganism, as shown in Fig. 1.

3.2. Ability to form biofilms

In the biofilm formation test on PS, 41.6% of the isolates were characterized as weak biofilm producers and 58.4% as non biofilm producers (Stepanović et al., 2007). There were significant differences among the isolates ($p < 0.05$), as shown in Fig. 2.

The highest biofilm forming ability was shown by SAS10, followed by SAS9 and SAS11. There were no significant differences ($p < 0.05$) between them, but significant differences between the three highest biofilm producers and the others isolates were observed.

All the isolates also produced biofilms on SS surfaces at 25 °C (log CFU/cm^2), as shown in Fig. 3. Differences between the isolates were significant ($p < 0.05$).

The highest ability for biofilm formation was observed for SAS7 followed by SAS8, SAS6 and SAS1 with no significant differences ($p > 0.05$) between them.

The lowest ability for biofilm formation was observed for SAS5 and SAS4 with a significant difference ($p > 0.05$) between them.

3.3. Presence of icaA, icaD and classical enterotoxin genes

The *ica*A gene was found in 66.6% of the isolates and *ica*D in 58.4%. The presence of both genes was observed in 58.4% of the isolates. Four isolates (33.4%) did not have either of these genes (*ica*A and *ica*D), but they were able to form biofilms.

The enterotoxins genes A and B were found in 33.4% and 16.6% of the isolates, respectively. None of the isolates had genes coding enterotoxins C, D and E. SAS8 had the genes for both enterotoxins A and B. These results can be seen in Table 3.

3.4. Antimicrobial resistance test

The isolates were resistant to ampicillin (66.6%), penicillin (66.6%), tetracycline (33.4%), erythromycin (8.4%) and sulphonamides (8.4%). Furthermore, intermediary resistance to gentamycin (8.4%) was observed. Multi-resistance to erythromycin, tetracycline, ampicillin and penicillin was observed in 8.4% of the isolates. These results are shown in Table 4.

4. Discussion

Handmade sweets are extensively handled manually and are not submitted to any subsequent thermal or anti-microbial processing. Thus, this kind of food is frequently contaminated by *S. aureus*. These microorganisms have the ability to form biofilms on both biotic and abiotic surfaces, and they are able to multiply on the skin and mucous membranes of the food handlers (Trabulsi and Alterthum, 2008). This study showed that 12% of the sweet samples were contaminated with *S. aureus*, which can indicates that there was improper handling and/or that equipment and surfaces were contaminated with biofilms formed by these bacteria.

Di Giannatale et al. (2011) evaluated different foods of animal origin, and found that 14% of the samples were contaminated with *S. aureus*. Vitale et al. (2015) evaluated milk, cheese, meat and ingredients for food preparation, and found that 10.5% of the samples were contaminated with *S. aureus*, and in 46% of these isolates at least one enterotoxin gene was present. Both authors point out that proper hygiene of the food handlers, as well as correct cleaning of the food preparation surfaces, should decrease contamination by this microorganism, minimizing the risk of disease.

It should be taken into consideration that with regard to biofilm formation on SS coupons, the result is expressed as the concentration of viable bacteria per cm^2. However, when biofilm formation was evaluated on PS microplates, the results of OD measures included the concentrations of both viable and dead cells.

The bacterial concentration found on a surface that is deliberated necessary for it to be considered a biofilm varies between authors. According to Andrade et al. (1998), a minimum of 10^7 cells per cm^2 is required for this purpose. On the other hand, Ronner and Wong (1993) and Wirtanen et al. (1996) consider a biofilm as 10^5 and 10^3 cells per cm^2, respectively. All isolates evaluated in this study gave concentrations above 10^7 CFU cm^{-2} for cells adhering to SS coupons. Therefore, it can be inferred that these isolates are

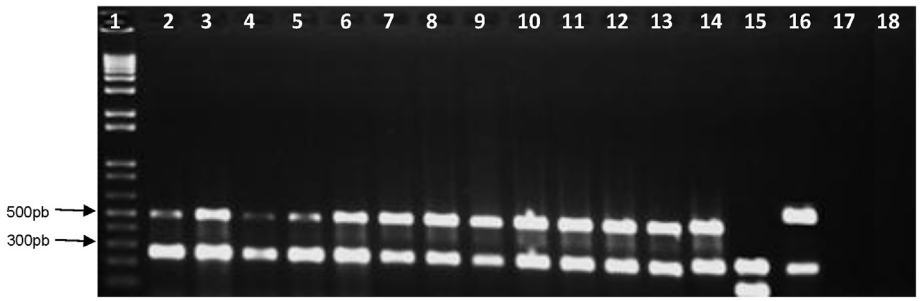

Fig. 1. PCR products obtained with *nuc* 1- *nuc* 2 (458 bp), *nuc* 5- *nuc* 6 (106 pb), *nuc* 7-*nuc* 8 (740 pb) and 16S rRNA (252 pb) primers.1) 1 Kb standard molecular weight Marker (1 Kb Invitrogen); 2–13) coagulase-positive Staphylococci isolated from handmade sweets; 14) positive control (*S. aureus* ATCC 25923); 15) *S. intermedius* ATCC 29663; 16) *S. hyicus* ATCC 11249; 17) negative control (*E. coli* ATCC 11229); 18) reaction control - sterile ultra-pure water.

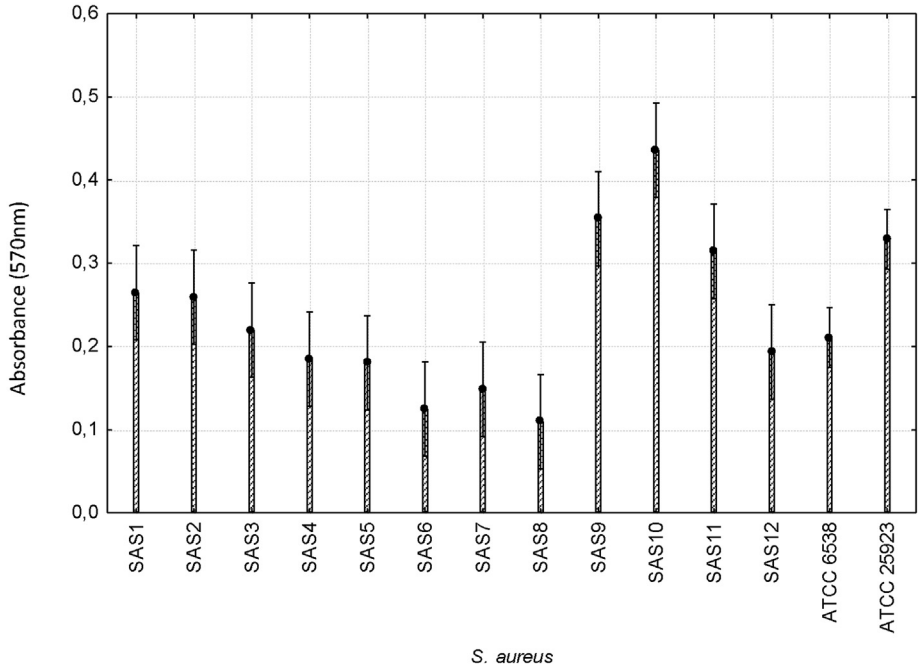

Fig. 2. Biofilm formation (PS surface) results for all of the tested *S. aureus* isolates (n = 12) cultured in TSB supplemented with 1% glucose at 25 °C for 24 h. The mean of three independent measurements is presented. Error bars indicate the standard deviation.

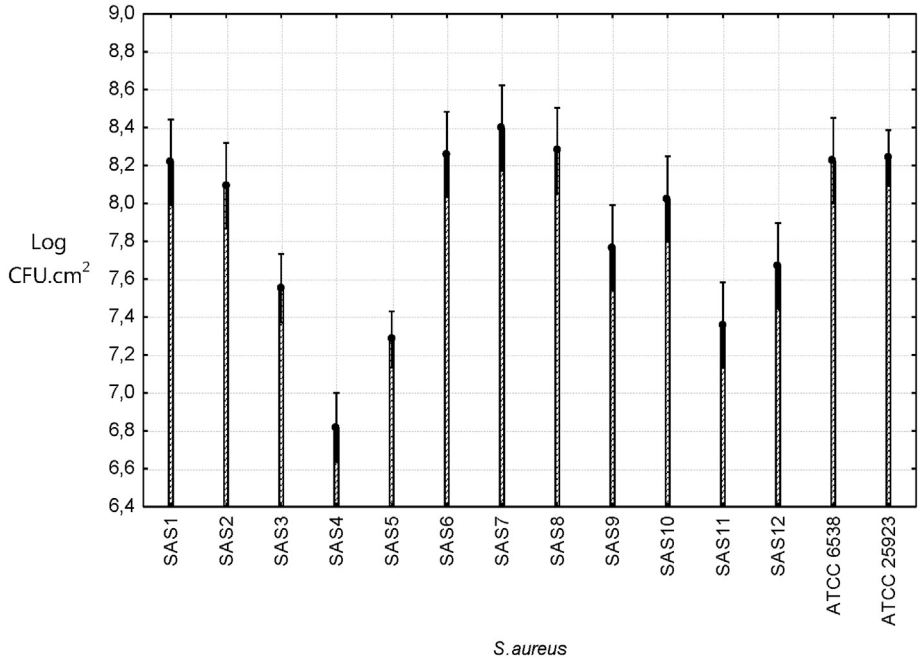

Fig. 3. Viable counts of *S. aureus* isolates (n = 12) cultured in TSB supplemented with 1% glucose at 25 °C for 24 h. The mean of duplicate independent measurements is presented. Errors bars indicate the standard deviation.

strong biofilm formers on SS surfaces (Andrade et al., 1998).

The presence of *ica*A and *ica*D genes indicates that the isolates have the genetic ability to form biofilms. However, the *ica locus* genes are not the only ones responsible for this, as isolates can use both *ica*-dependent and *ica*-independent mechanism for biofilm formation (Ferreira et al., 2012; Giaouris et al., 2015; O'Gara, 2007; Rode et al., 2007). In addition, studies have shown that there are variations in the expression of *ica locus* genes among *S. aureus* isolates. For example, when *S. aureus* is exposed to different temperatures, times and contact surfaces, different gene expression profiles can be observed (Atshan et al., 2013; Stanley and Lazazzera, 2004). As we observed in this study, 33.4% of the isolates formed biofilms independent of the presence of the genes of the *ica locus* (SAS4, SAS7, SAS8, SAS9), so it can be inferred that an *ica*-

Table 3
Presence of *ica*A and *ica*D genes, related to biofilm formation and presence of enterotoxins genes (A, B, C, D and E) in *S. aureus* isolated from handmade sweets.

Isolates	*ica*A	*ica*D	*sea*	*seb*	*sec*	*sed*	*see*
SAS1	+	+	+	−	−	−	−
SAS2	+	+	+	−	−	−	−
SAS3	+	−	−	−	−	−	−
SAS4	−	−	−	−	−	−	−
SAS5	+	+	−	−	−	−	−
SAS6	+	+	+	−	−	−	−
SAS7	−	−	−	−	−	−	−
SAS8	−	−	+	+	−	−	−
SAS9	−	−	−	−	−	−	−
SAS10	+	+	−	−	−	−	−
SAS11	+	+	−	+	−	−	−
SAS12	+	+	−	−	−	−	−

independent mechanism exists.

In this study we used a temperature of 25 °C to simulate the temperature of the processing environment for handmade sweets. Rode et al. (2007) found that temperatures below the optimum temperature for *S. aureus* multiplication (37 °C) increased biofilm formation and so expanded the risk of contamination of food with isolates that have the ability to form biofilm. The same authors found that in *S. aureus* isolated from food, there were strong biofilm formers in similar conditions to those found in the food industry (20 and 25 °C/24 h). Similar results obtained by Vázquez-Sánchez et al. (2014) showed that all *S. aureus* isolates from fishery products (twenty six isolates) had a high capacity to form biofilms, with cell counts above 10^4 CFU cm^{-2} after 5 h at 25 °C on a SS surface, implying that the food processing may have exerted a selective pressure. The same was also observed in this study, in which 100% of the isolates were able to form biofilms at 25 °C on SS surfaces. This study does suggest that factors, such as temperatures below the optimal temperature for the growth of these microorganisms, and selective pressure during processing, may have positively influenced biofilm formation and the consequent contamination of handmade sweets samples.

The gene for enterotoxin A was prevalent (33.4%) among the isolates, pointing to a high risk of these isolates causing staphylococcal food poisoning (Kérouanton et al., 2007). Besides the presence of the enterotoxin A gene, 16.6% of the isolates also carried the enterotoxin B gene.

The prevalence of these two toxins was also examined by other authors when they evaluated *S. aureus* isolated from food. Song et al. (2015) found that 5.6% of the isolates from raw and processed foods carried the enterotoxin A gene (*sea*) and 3.5% carried the enterotoxin B gene (*seb*). Similarly, Zhang et al. (2013) found the *sea* gene in 24.1% of the isolates and the *seb* gene in 4.2% of the isolates from raw meat products, cooked or baked meat products, dairy products, quick-frozen foods, ready-to-eat vegetables, beverages, aquatic products, bean products and bee products. These authors also found that 20.5% of the isolates carried the *sed* gene, 6.8% carried the *sec* gene and 0.6% carried the *see* gene. In contrast, in this study, the isolates did not carry any of these genes.

Even without the *sec*, *sed* and *see* genes, these isolates have enterotoxigenic potential because they may possess the *sea* and *seb* genes. The *sea* gene is the most commonly reported in contaminated foods and also considered the main cause of staphylococcal food poisoning worldwide, while the *seb* gene is known for promoting more severe poisoning than other enterotoxins (Argudín et al., 2010; Carfora et al., 2015).

The majority of the isolates had the *ica*A and *ica*D genes, responsible for facilitating the formation of biofilms. However, this is not the only mechanism involved as there were some isolates that did not present those genes and they were still able to form biofilms on SS, e.g. isolate SAS8. This isolate had both the enterotoxin A and B genes, and according to Da Silva et al. (2005), the presence of these two genes in the same isolate increases its toxigenic capacity. Moreover, it was able to form biofilms on both PS and SS, even without having the *ica*A and *ica*D genes. This result confirms the ability of *S. aureus* isolates to form biofilms independent of the *ica locus* (Rode et al., 2007).

Examining antimicrobial resistance, it was found that resistance to β-lactamics (penicillin and ampicillin) predominated among the isolates, followed by tetracyclines (tetracycline), macrolides (erythromycin) and sulphonamides. All the isolates were resistant to at least one antimicrobial agent. Resistance to penicillin and ampicillin is highest in *S. aureus* and can reach frequencies above 70% (Unakal and Kaliwal, 2010). In this study, the level of resistance to these antimicrobials was 66.6%.

We observed that 8.4% of the isolates were multiresistant to erythromycin, tetracycline, ampicillin and penicillin. Tan et al. (2014) evaluated *S. aureus* isolated from the hands of handlers, and found resistance levels to ampicillin and penicillin, and a low multidrug resistance level (5.41%), similar to the one obtained in this study.

Accordingly, the resistance levels found in this study can be explained by the indiscriminate use of antimicrobials in humans treating diseases, which is a worrying trend, as these isolates also have the ability to form biofilms. Under certain circumstances, such as the ones encountered during biofilm formation, the bacteria may encounter sublethal doses of drugs, so contributing to the emergence of resistant isolates.

We found *S. aureus* among the CPS isolates from handmade sweets produced in Pelotas, Brazil. The isolates had virulence

Table 4
Mean zone of inhibition and percentage of antimicrobial resistance of *S. aureus* isolated from handmade sweets.

Antimicrobial isolates	Mean zone of inhibition (mm)												
	1	2	3	4	5	6	7	8	9	10	11	12	Resistance (%)
Gentamicin (10 μg)	26.5	25.5	23.5	24.5	14	26	27	26	32	33	24	26	0
Chloramphenicol(30 μg)	26	26.5	25.5	28	28	31.5	31	30	28	30	28	28	0
Clindamycin (2 μg)	31	29	30	31.5	33	34	30	25.5	33	31	29	27	0
Sulfonamide (300 μg)	35	33	31.5	10	34.5	19.5	22.5	26	34	30	18	25	8.4
Ampicillin (10 μg)	49	49	16	17	16.5	21.5	21	22.5	36	35	20	22	66.6
Ciprofloxacin (5 μg)	33.5	34	32.5	35	30	37	33.5	32.5	31	32	35	32	0
Erythromycin (15 μg)	26	27.5	28.5	29.5	26	35	29	10	31	30	28	29	8.4
Vancomycin (30 μg)	26.5	20.5	20	21	21.5	19.5	21	20	21	21	20	20	0
Tetracycline (30 μg)	31.5	30.5	29.5	28	29	29.5	29.5	10	7	8	8	29	33.4
Penicillin (10 U)	49.5	51	15.5	16	16	20	21.5	22	35	37	20	22	66.6
Cephalothin (30 μg)	43.5	43.5	30	30.5	13	33.5	34.5	36	41	40	32	36	8.4
Cefoxitin (30 μg)	31	34.5	29	28	31.5	30	22.5	28.5	29	29	30	27	0

potential, since half of them were enterotoxigenic, carrying the enterotoxin genes often involved in outbreaks of staphylococcal food poisoning. In addition, the ability of all the isolates to produce biofilms highlights the risk of these potentially virulent microorganisms persisting in the food manufacturing environment.

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